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Equilibrium Testing of Rat Tail Tendon: An Analysis of the Viscoelastic Properties of Collagen Under Different Strain Points

Joshua C. Witt
Rose-Hulman Institute of Technology, wittjc@rose-hulman.edu

Equilibrium Testing of Rat Tail Tendon: An Analysis of the Viscoelastic Properties of Collagen Under Different Strain Points

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Final Examination Report

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EXAMINATION COMMITTEE:

	Thesis Advisory Committee	Department
Thesis Advisor:	Glen Livesay	BBE
	Renee Rogge	BBE
	Simon Jones	ME

PASSED X **FAILED** ____

Abstract

Witt, Joshua, Colpe

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Equilibrium testing of rat tail tendon: An analysis of the viscoelastic properties of collagen under different strain points.

Thesis Advisor: Dr. Glen Livesay

Instantaneous tensile testing and stress-relaxation testing are forms of mechanical testing used to determine the elastic and viscoelastic properties of biological tissue. Equilibrium testing is a form of testing that combines both of these testing approaches at different strain points to determine the elastic properties of a material and also assess their viscoelastic properties in the same test. This testing method is commonly used on highly viscoelastic materials such as cartilage but has never been fully described in dense collagenous materials such as tendon or ligament. This analysis utilizes different strain points selected to capture the classic non-linear behavior of stress-strain curves at lower strain values of dense collagenous materials. During an equilibrium test, the material is loaded instantaneously to the first strain point and held there, such that the material then experiences stress-relaxation. After a pre-determined holding period, the material is then pulled by the same elongation to the second strain point and allowed to experience another stress-relaxation. In this experiment, this was conducted for six cycles for each specimen. Strain points were spaced equally by using the transition point as a basis for analysis to include three points within the toe region, one at the transition point, and the last two in the linear region.

During these stress-relaxation tests at different strain points, the material was expected to experience an exponential growth during the elastic pulls and then exponential decay during stress relaxation. It was found that both of these parameters were highly dependent on the strain states, indicating a potential additive model for viscoelasticity as well as casting some doubt on the validity of superposition as an assumption of collagenous materials. It was also found that the equilibrium modulus was different between the toe and the linear regions. Based upon these findings, the modulus determined for rat tail tendon using the equilibrium testing approach did display a similar pattern to moduli determined using the instantaneous tensile method.

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β_1 (pull) The slope of the pull for the equilibrium curve. This value is adjusted for each pull.

β_1 (hold) This is the asymptotic value for the stress-time model normalized data for each hold. This value adjusts for each hold.

β_2 (hold) This is an adjustments value for the hold exponential decay model. This value adjusts for each hold.

β_3 (hold) This is the rate of exponential decay for each normalized hold model. This value adjusts for each hold.

H The indicator value for each hold. This is a binary value that will correspond to a linear combination with the beta values for each hold.

P The indicator value for each pull. This is a binary value that will correspond to a linear combination with the beta values for each pull.

ε This is the representation of strain. This value is calculated by dividing the elongation by the initial length of the material.

ε_t The total strain of a viscoelastic material.

ε_s The strain that results from the elastic component of a material.

ε_f The strain that results from the viscous component of a material.

σ	This is the representation of stress. This value is calculated by dividing the cross-sectional area of the material by the force that is being exerted on the material.
t	This value represents time. This value is the independent variable of the stress-time curves.
η	Viscoelastic constant, accounts for the rate of change in stress with respect to strain for the fluid component of a material.
E	The elastic modulus, accounts for the rate of change in stress with respect to strain for the solid component of a material.

Glossary

Collagen - This protein is one of the most common structural protein of connective tissue and almost all other tissues. This structure is extremely strong and makes up most of the material of tendons and ligaments.

Crimp - This is a microstructure of tendon and ligament where the collagen fibers appear to be wave-like. This structure is what gives rise to the toe region of the stress-strain curves of tendon, ligament and most other highly collagenous materials. This is due to this structure being straightened out within this region.

Elastic - This is a material state of a material where it can undergo deformation under loading. By this the material undergoes small changes in length with respect to the same direction of the loading.

Equilibrium testing - The utilization of multiple loadings to certain strain points to understand the additive effect of viscoelastic properties over strain points towards an equilibrium modulus.

Fascicle - A unit of tendon that is below that of the entire fiber. This structure is approximately 0.15-0.35 mm in thickness and form bundles to form tendon fibers. These structure are divided by connective tissue or fascia.

Instantaneous testing - This form of loading assumes that the elastic characteristics of a material can be exhibited when the material is only pulled once and as quickly as possible. This is usually characterized by strain rates that are very high.

Strain - This is the normalization of the elongation of a material that is experiencing loading by dividing the elongation of the material by its initial length prior to loading.

Stress - This is the force that is exhibited on a material that is normalized by dividing by the materials cross-sectional area. The base unit of stress is the mega-Pascal ($MPa=N/mm^2$).

Superposition - In a linear system, the summation of the responses of individual inputs is equal the net response of the system.

Tangent Modulus - This term is used to qualify the rate of change of the stress of a material with respect to the strain. This rate refers specifically to stress-strain curves of materials.

Transition point - This is the point when the crimp of the tendon has been completely elongated and now the material experiences a linear stress-strain behavior.

Tropocollagen - This is the basic molecular structure of collagen fibers. This structure consists of three alpha-helices that form a triple helix. These structures are held together via intramolecular covalent bonds such as disulfide bonds. The strength of these structures lend to the bonding and the counter-rotating nature of this helix compared to that of the alpha-helices. This structure has a left-handed twist while the alpha-helices form a right-handed twist.

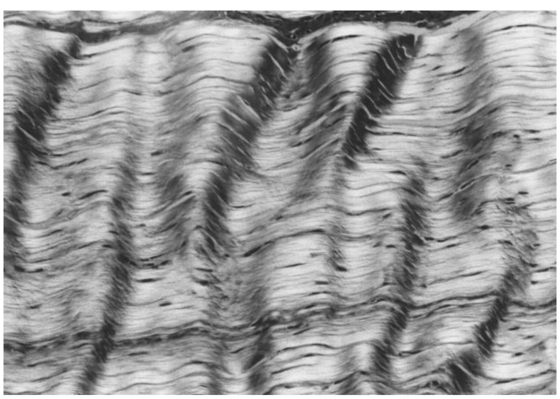
Viscoelasticity - The ability of materials that exhibit both solid and fluid behavior. That is the instantaneous and time dependent mechanics of both types of materials. This is usually characterized by deforming instantly with respect to a load and also by continuously deforming on a similar load over time.

1 Introduction

1.1 Collagen

Collagen is a material that constitutes a major portion of biological tissues. This material is comprised of a multilayer hierarchical structure gives it high tensile strength [1, 2]. This structure starts at the molecular level that is tightly wound due to a high prevalence of glycine, the smallest amino acid, at every third amino acid in the alpha helical structure that has a right handed turn. Three of these molecules are wound together to form a triple helical structure of tropocollagen which has a left handed turn. This counter rotating structure from the alpha-helices and the tropocollagen is what gives collagen its strength [3].

1.2 Tendon Structure

a)


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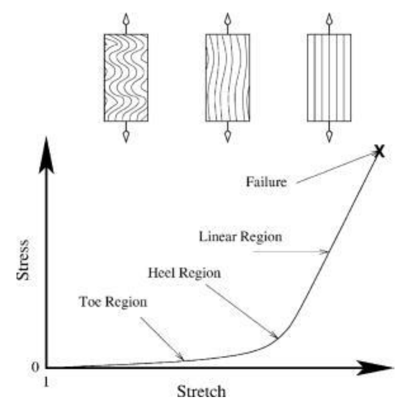


Figure 1: a) A microscopic view of the crimp of the microfibrils found in tendon [4]. This structure gives the tendon the laxity in low strains. **b)** The mechanical response while showing the state of the crimp structure. At low strain values, the crimp is relatively the same but at a point (graphic refers to heel but for intent of this paper it will be refered to as the transition point) is when the crimp is straightened and linear elastic properties begin to show [5].

At higher levels of the structure of highly collagenous materials, there is a wavy pattern in the material known as crimp. A microscopic image of crimp can be seen in Figure 1. This is most commonly seen in connective tissues such as tendon and ligament which primarily have a function of resisting loads in tension to facilitate muscle action or to stabilize joint articulation. Crimp gives rise to the global mechanical properties of tendon and ligament. The pattern of loading and a comparison with the length of the crimp can be seen in Figure 1b. As the materials are initially stretched they exhibit nonlinear behavior as the crimp is stretched out [6]. Once the crimp has been fully elongated, the mechanical response of collagen is approximately linear. The point at which the collagen fibers have been straightened and the crimp has been straightened out is known as the transition point.

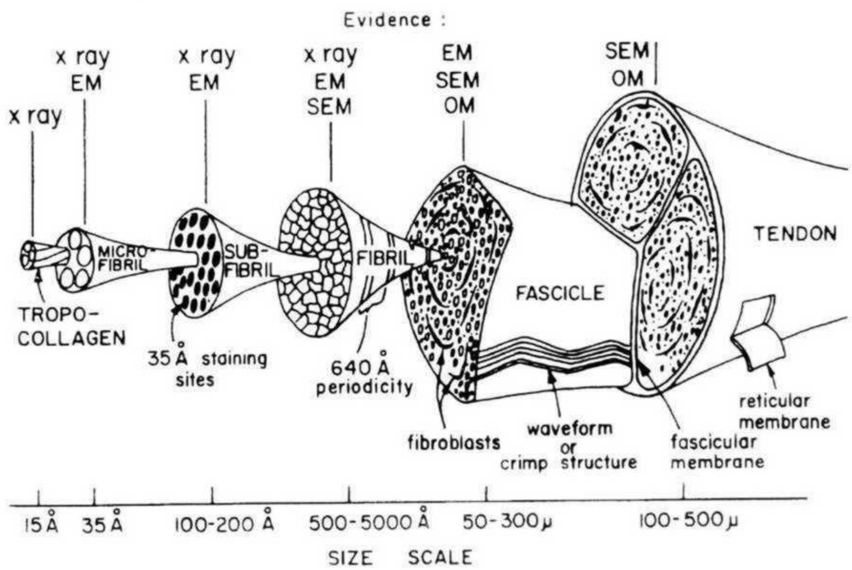


Figure 2: Structure of tendon from tropocollagen to entire structure of the tendon [7].

Tendon and ligament have a very organized hierarchical structure which can be seen in Figure 2. Tendons act to connect muscle to bone while ligaments are used to attach bone to bone. Tendons act to transmit force for stabilization or for movement of the bone via skeletal muscle. Each sub-segment is separated is contained via connective tissue and is separated by interstitial fluid. The crimp structure is typically seen at the microscopic levels with the wavy structure seen in the microfibrils of tendon. In order to demonstrate the mechanical properties of collagen as a factor in tendon, it is better to use sub-segments of the entire structure. That is why in this study, fascicles will be used for mechanical testing.

1.3 Mechanical Response of Tendon Under Tensile Loading

When referring to elasticity, one must consider what the type of loading is being applied to the material. This is especially true because materials, especially those of biological origin, exhibit distinct behavior under instantaneous loading and under extended time loading. This usually involved two separate tests to measure the specific properties at these time frames. Even under testing long periods of loading, there is an apparent instantaneous response of the material as seen by a large impulse at the start of the test. The remaining portion is described simply as the decay due to viscoelasticity and is typically described by an exponential decay model. This incorporates a multiplicative term η for the viscoelastic response and the term E which represents the elastic modulus which is determined through instantaneous testing. The height of the initial impulse from the instantaneous fraction can also observed.

$$\sigma = \sigma_o e^{-\frac{E}{\eta}t} \tag{1}$$

1.4 Traditional Instantaneous Testing

The most commonly used form of testing is the instantaneous tensile testing paradigm to measure the material properties that occur in this time frame, as shown in Figure 3. The controlled parameter is the rate at which the material is being placed under strain relative to time, or the strain rate of the test. This is shown through the slope of the graph on the left of Figure 3. The resultant behavior is seen in the stress-strain plot as seen on the right graph of Figure 3. For highly dense collagenous materials such as tendon or ligament, there are three distinct regions that are found: toe region, transition point, and linear region. As described earlier, this is primarily due to the elongation of the crimp structure found in the micro-fibrils of the tendon.

The tangent modulus is the property that is observed from this testing paradigm as the slope of the linear region. However, when observing the viscoelastic properties of tendon in the toe region that is distinctly non-linear, would modeling the characteristics of a distinct region be tenable?

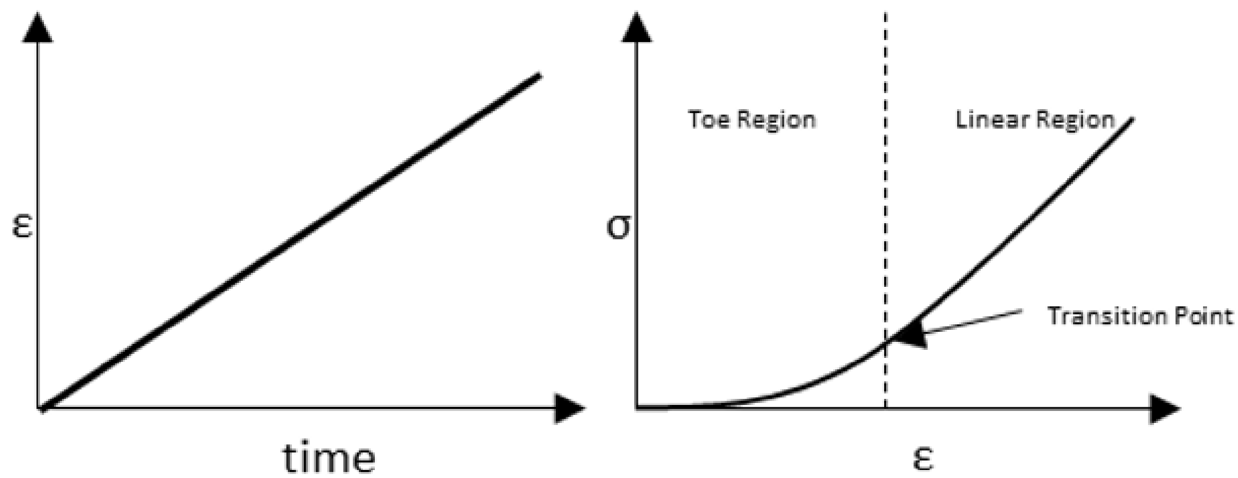


Figure 3: The testing paradigm for the instantaneous loading of a material. The controlled parameter is the rate at which the material is elongated, or the strain rate of the material, is seen on the left. On the right is the resultant stress-strain curve with the distinct toe region, transition point, and linear region. This is observed because of the elongation of the crimp structures at the micro-fibril level of the tendon.

1.5 Viscoelastic Characterization-Traditional Approach

While the material properties of tendon and ligaments are primarily governed by collagen, other components also contribute to the material properties [8-10]. Since this material is comprised of biological tissue [10], and therefore water, there are viscoelastic properties that must be considered when analyzing how a material reacts to specific forms of loading. Most viscoelastic materials usually behave under the Maxwell model that combines both the linear and nonlinear properties of the material. This assumes that there is an elastic or instantaneous portion of the material while also considering a viscous or delayed reaction to a given load. These properties are actively shaped by components of the material itself or by external factors [11].

In order to fully understand the properties of ligaments and tendon, and by proxy collagen, it must be understood how this material reacts under different forms of loading. When testing for the material properties of highly collagenous materials such as tendon, it is commonly assumed that these materials should be tested in an instantaneous fashion in order to determine their elastic properties [12]. However, this form of testing may not be valid due to the viscoelastic nature of tendon and how differing structures within tendons and ligaments behave within this material. These differing structures allow for the tendon fascicles to be either an energy storing or non-energy storing [13, 14].

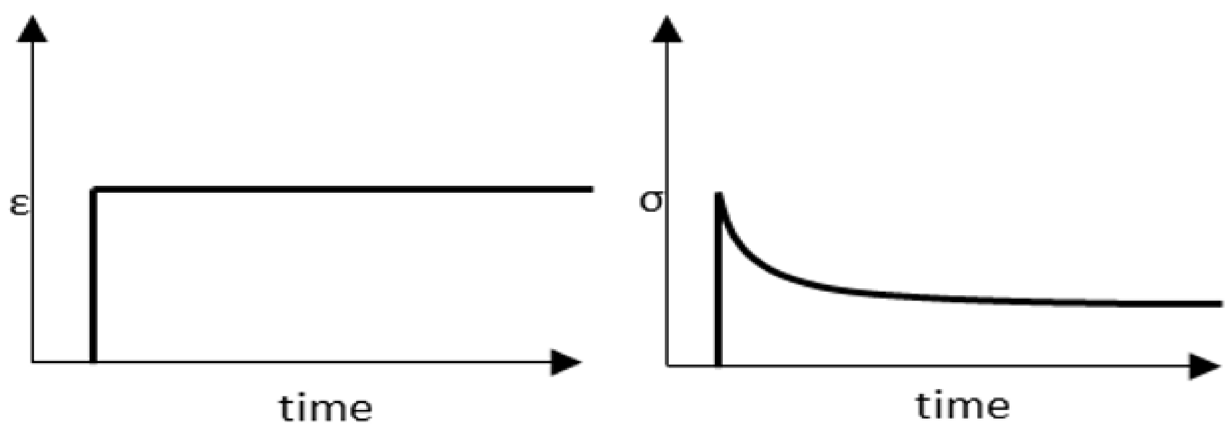


Figure 4: Shown above is the stress-relaxation testing paradigm where a material is loaded to a given length or strain and the stress over a given time is observed. The instantaneous portion of the material is seen by the initial stress impulse and the viscoelastic decay or relaxation of the material is observed.

One of the more traditional means of measuring the viscoelastic properties of biological samples is through the stress-relaxation test. This test demonstrates the amount of stress decay that results when a material is held at a given strain value for an amount of time. The impulse seen in the beginning of the stress-time plot in Figure 4 (right) is the instantaneous elastic response. As the material is held at this length, it begins to “flow” and relax resulting a lesser stress that is needed to maintain the material at this given strain value for the amount of time.

This decay is commonly modeled as an exponential decaying function, as seen previously. This test is much more commonly performed because the constant parameter, strain, is much easier to control than stress because of force calibration of the machine and the cross-sectional area calibration for the sample that would needed as well.

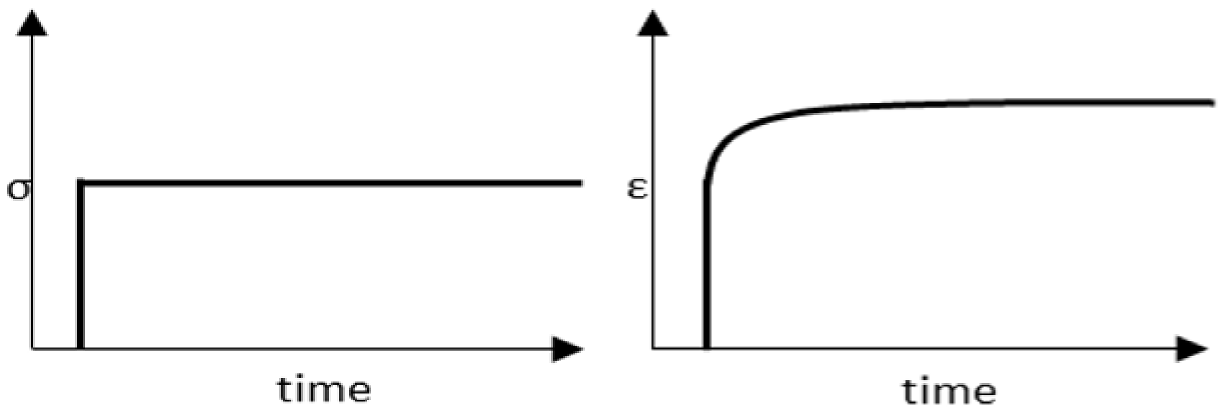


Figure 5: Shown above is the creep testing paradigm where the material is loaded to a specific force or stress and the strain is measured over a given time frame.

The other test that is used to measure the viscoelastic properties of a biological material is the creep test. This test is the opposite of the stress-relaxation test, wherein the stress is held constant and the elongation of the material is measured over time. This can be seen in Figure 5 with the stress being held at a constant value and the strain of the material being measured. In this case however, the material undergoes the opposite of the stress-relaxation in terms of the response after the initial impulse. This form of testing is usually done in compression with other biological materials such as cartilage. Those materials are typically kept at a constant cross-sectional area and demonstrates how long it would take for this type of material to “settle” after it has been placed under loading.

1.6 Equilibrium Testing

The equilibrium test provides a means to combining both the instantaneous elastic and viscoelastic tests into one to evaluate both properties and the interplay between them. The controlled variable in this testing parameter is pulling to a predesignated strain point and holding at that point for a predesignated amount of time as seen in the plot on the left in Figure 6. The resultant curves that are produced are the stress-time (top) and stress-strain (bottom) of Figure 6. The stress-time curve demonstrates how the viscoelastic properties may change over the strain points. Meanwhile the stress-strain curve can be further evaluated during each pull and also producing a composite equilibrium curve with the minimum value of the hold. The plots allow for a greater understanding of how the material behaves instantaneously as well as knowing how the "what remains" portion changes across the strain points. This form of testing has been done in both tendon [15, 16], as well as in cartilage [17].

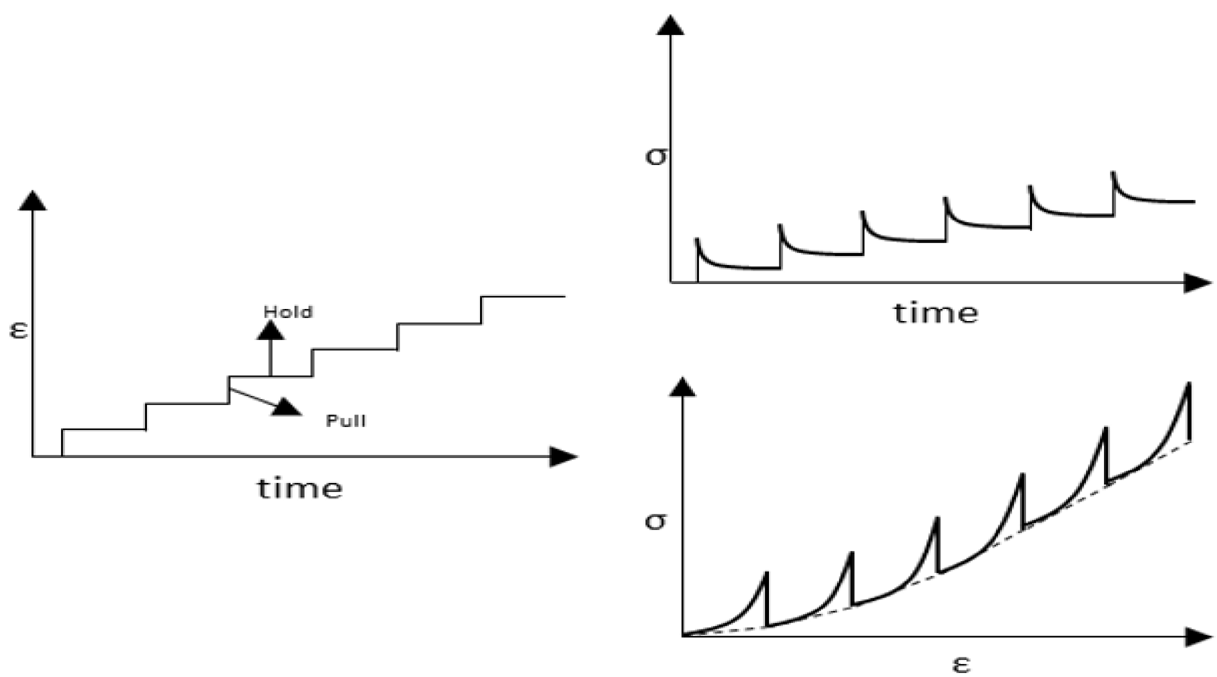


Figure 6: Above is the testing paradigm for the equilibrium test. The plot to the left demonstrates the controlled variable, the strain being pulled at intervals and held at a strain point for a designated amount of time. The plots the right are the expected outputs of the test, the stress-time (above) and stress-strain (below). The equilibrium composite curve produced from the stress-strain minimum values from each hold is shown by the dashed line.

However, this form of testing has not been conducted on highly collagenous tensile materials such as tendon. In order to accomplish this form of testing, rat-tail tendon fascicles will be used in both instantaneous and equilibrium testing. These tests will be compared to each other, and the values from the instantaneous tests will be used to determine the strain intervals that are to be used for the equilibrium tests. The equilibrium tests will further analyze the tendon through six pull-hold cycles for each sample for the repeatability of the stress-strain and stress-time reactions across the strain points.

This testing paradigm will be used in order to determine if a constant elastic modulus should be used for modeling this type of behavior. Also, this test will be used to determine how the viscoelastic properties may change over strain intervals. If there is an effect that may be found, it may undermine the principle of superposition that is needed for our current understanding of the interplay of elasticity and viscoelasticity. With greater understanding of how viscoelastic properties behave over different strain points, would a model with a single multiplicative variable to describe the viscoelastic properties be needed or would an additive one that accounts for strain be needed?

2 Methods

2.1 Sample Preparation

For this procedure, rat tail tendon was used. The samples frozen rat tails that were thawed for approximately twenty minutes prior to excision. Once ligaments were excised, they were to be further segmented into fascicles and cut into segments of 3-4 inches in length. Once excised, samples are placed into deionized water prior to measurement and experimentation.

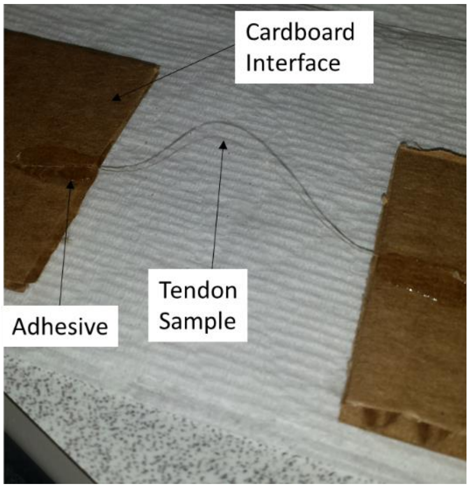
Once the samples were selected for analysis, they were placed into the testing fixture. This device was constructed to allow the samples to experience tension throughout the experiment but reduce the stress concentration at the clamp sample interface [18]. To reduce the stress at this interface, cardboard was utilized [18]. In order to firmly secure the sample, both ends of the sample were affixed to sand paper via adhesive. Once the sample is secured within the device, it is kept wet before dimensional measurements are taken.

The samples were assumed to have an approximately square cross-sectional area. The width of the samples was measured under a microscope with the use of a micrometer. The initial length of each sample was measured when placed into the loading cell prior to pulling the sample. These values were to be used for the calculation of the stress and strain values. Added consideration of cross-sectional area deformation was taken into account by assuming constant volume of the sample and using the current length at a given point to determining the cross-sectional area.

2.2 Testing Procedure

In order to determine the transition point that was used as the basis for equilibrium testing, six samples are placed under tensile testing until failure. An Instron 5966 10kN loading cell was used that utilizes Bluehill 3 software for testing procedures. All samples are to be tested at a strain rate of 2.5mm/sec and a sampling of 10 Hz. All samples were kept wet prior to testing but were not interfered with during testing.

a)



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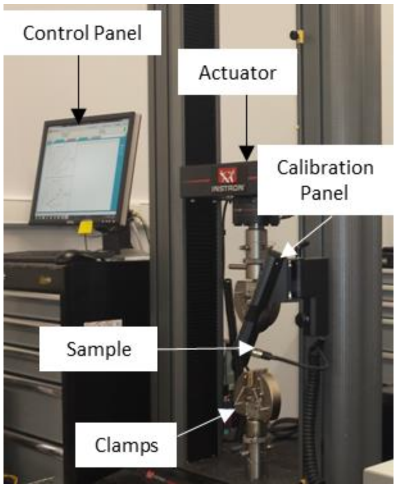

Figure 7: The testing setup for both the instantaneous and equilibrium testing paradigms of rat tail tendon. a) The mounting setup for the interface of the tendon to load cell showing the tendon sample, the adhesive, and the cardboard interface. b) The loading cell set up for all of the testing showing the control panel, calibration panel, actuator, clamps, and where the sample would be placed for loading.

2.3 Instantaneous Testing

For the instantaneous testing procedure, samples were placed under tension until failure. Six samples were used and the end of the toe region as well as the tangent moduli after each pull were calculated.

2.4 Equilibrium Testing

Once the instantaneous tests were conducted, the transition points were used as a basis for the equilibrium strain points. This was to allow for three points to be tested within the toe region of the samples and three to be conducted in the linear region of the samples. The third point of the toe region is to be the transitional point. It was found that the transitional point occurs at ~6% strain and therefore, the strain points for relaxation are to be held at 2, 4, 6, 8, 10, and 12% strain. Each hold is to last twenty minutes to allow for the material to equilibrate over time.

Once the load and elongation data was collected, it was further processed through time averaging. The transition to each strain point (the pull) was left unaltered. However, the holding points for each sample were time averaged on varying scales. For the first thirty seconds of data, intervals of five seconds are averaged together for both time and load. After this first segment, the data is then averaged across intervals of sixty seconds for the remainder of the pull. After the data has been filtered through averaging, the time, elongation, and load are translated into minutes, strain and stress respectively. The maximum values of the pull and the minimum values of the pull was used for analysis of the equilibrium and elastic composite curves.

After the data was processed, it was further translated into normalized values for each hold and pull. For each hold, the start time was subtracted by the instantaneous time value and each value of stress was divided by the initial value. For each pull, the starting strain was subtracted from the instantaneous strain and the initial stress of each pull was subtracted from the instantaneous stress. These values were used for a statistical analysis of the behavior of each pull and each hold at each strain point.

2.5 Statistical Testing

For a statistical analysis, nonlinear models were used in order to determine the variance of the coefficients across holds and pulls. An exponentially decaying function with indicators for each holding point were used as described below:

$$\sigma = (\beta_1 + \beta_{1i}H) + (\beta_2 + \beta_{2i}H)e^{-(\beta_3 + \beta_{3i}H)t} \tag{2}$$

This function displays asymptotic behavior (β1) as time approaches a limit and allows for rate (β3) adjustments (β2) according the data. The first coefficient is the allowance of error and population variance as well as the first holding adjustment. The H variable is a binary indicator for each hold from two to six. A subgroup estimation procedure was used for each hold to estimate the starting values for each coefficient.

For the pull analysis the following model was used to determine the variance of the parameters:

$$\sigma = \left(\beta_1 + \beta_{1j}P\right)\varepsilon + (\beta_2 + \beta_{2j}P) \tag{3}$$

The first term (β1) is change of stress across strain with adjustments to this value as a result of different holding patterns with adjustment (β2). P is a matrix of indicator values used for each pulls two through six. J is an index for the adjustment coefficients that corresponds to the indicator values of holds two through six.

These coefficients were placed under a parameter test to determine if the initial mean of the population and adjustment due to holding are non-trivial. This was further expanded to include whether the adjustments are equal to themselves and to determine if there is relation between the strain points. For this analysis, a 95% confidence interval (α=0.05) will be used to determine significance. All statistical analysis is to be conducted in the R/Rstudio package.

3 Results

3.1 Instantaneous testing

In order to analyze the equilibrium testing, strain had to be defined relative to the transition point of rat-tail tendon. This was done through tensile testing of six specimens at a strain rate of 2.5mm/sec. Figure 8 is the compilation of the stress-strain relationship of the instantaneous testing. There appears, for the most part to being a clear linear and nonlinear region (toe) across all of the samples. The transition strain and tangential modulus of the linear region are reported in Table 1. The mean tangent modulus for the six samples collected is 3.23±4.02 GPa while the mean transition strain is 0.06±0.027. With this data collected the holds for the equilibrium test are to occur at intervals of 2% strain to allow for three samples to occur in the toe region and three to occur in the linear region.

Table 1: Values of the tangential modulus and transitional strain of samples used for instantaneous testing at 2.5 mm/sec.

Sample	Moduli (GPa)	Transition strain
1	1.06	0.05
2	6.08	0.057
3	1.82	0.06
4	3.24	0.072
5	3.17	0.077
6	3.99	0.06
mean	3.23±4.02	0.06±0.027

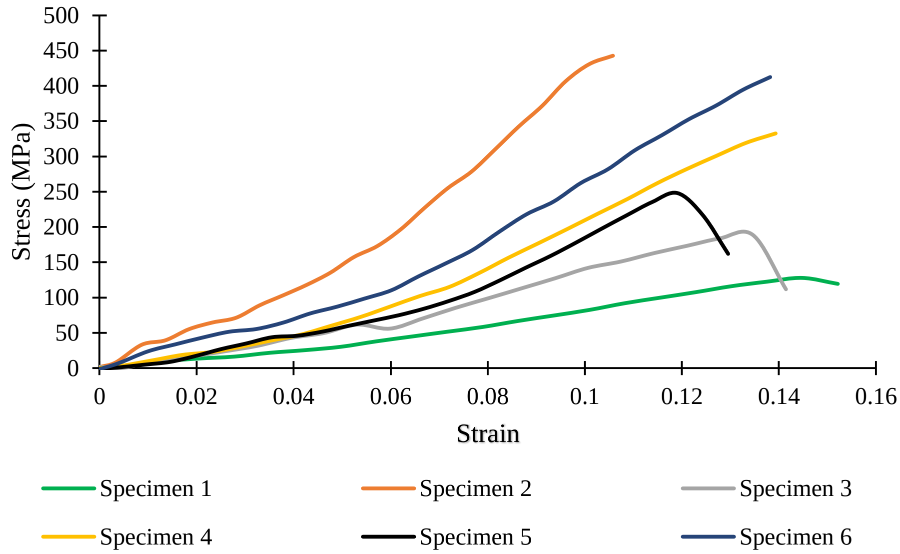

Figure 8: The instantaneous stress-strain curves for six samples (as seen in legend) tested at a strain rate of 2.5mm/sec. The specimens were tested until failure in uniaxial tension. There is a distinct linear and toe region for most of the Figures. The tangential modulus and transitional strain are for all of these are tabulated in Table 1.

3.2 Equilibrium testing

With the strain intervals defined at 2%, the equilibrium testing occurred at the same strain rate for each pull and each hold to last 20 minutes. It was expected that each pull would demonstrate an exponential like characteristic between the stress and the strain values. However, with the aid of a reference curve, this is not the case and the stress-strain curve instead demonstrates more of a linear or logarithmic relationship. This relationship is displayed in Figure 9 which depicts the nature of the curve for sample three. The curves for the other samples can be found in the appendices. The stress-time relationship is displayed in Figure 10 for sample five. This curve displays characteristic viscoelastic behavior of the material with an impulse in the stress for every pull and exponential decay of the stress over time. The stress-time values for the other samples can be found in the appendices.

A comparison of the equilibrium and elastic composite curves can be found in Figure 11 displaying the values found for sample five. The equilibrium curve takes the minimum stress values for each hold at each strain interval and the elastic curve is a series of the maximum stress values for each pull for each strain interval. Both of these curves demonstrate a nonlinear toe region and a linear region. There appears to be a difference in the offset of the toe region between the equilibrium elastic regions but with similar linear regions. The values for comparison of the elastic and equilibrium curves can be found in Table 2. The Figures for the other samples can be found in the appendices.

Table 2: The slopes found at each strain point for the equilibrium curve and the elastic curve. These are the mean across the seven samples.

Period	Equilibrium Slope (MPa)	Elastic Slope (MPa)
1	325.73	820.29
2	671.10	1324.62
3	772.09	1025.32
4	1152.56	1335.55
5	1454.69	1655.40
6	2119.09	2250.86

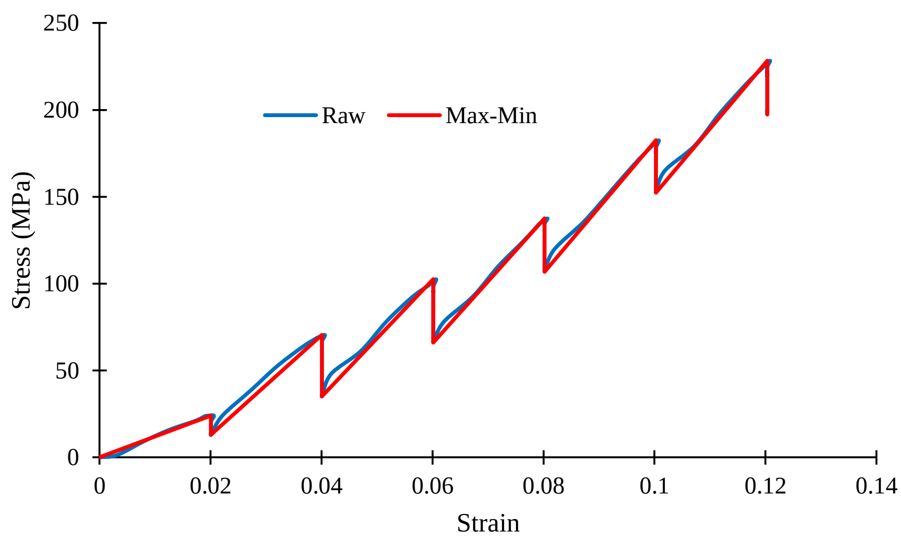

Figure 9: The equilibrium stress-strain curve with six strain points at intervals of 2% strain. The test was conducted in tension at 2.5mm/sec uniaxial in a loading cell. The above Figure is of sample three the depicted raw data (blue) and composite curve of the minimum values of the hold and maximum values of the pull (red). These curves demonstrate the initial stiff response of each pull and the limit of the modulus between each pull as the strain increases.

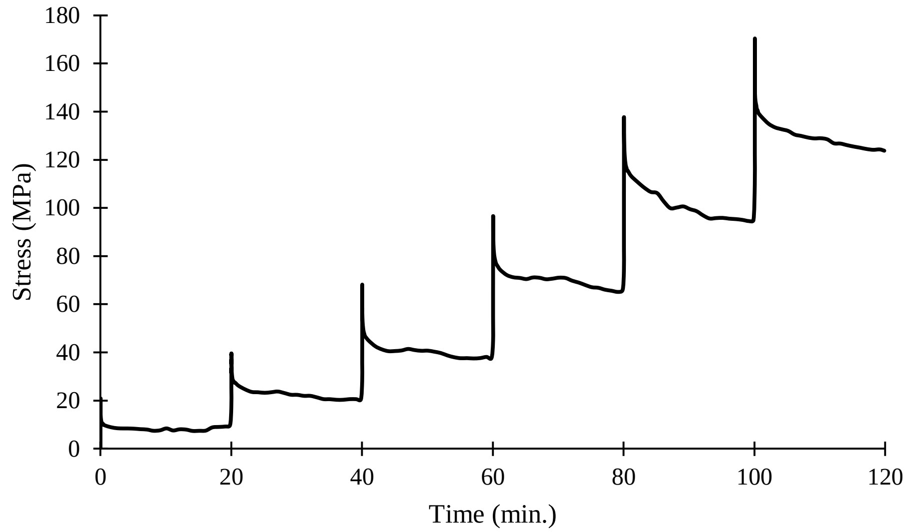

Figure 10: The equilibrium time-stress curve with six strain points at intervals of 2% strain. The test was conducted in tension at 2.5 mm/sec uniaxial in a loading cell for pulls and the holds occurring at each strain point for 20 minutes. The Figure above depicts the curve for sample five. This Figure displays the viscoelastic response of rat tail tendon across six different strain points.

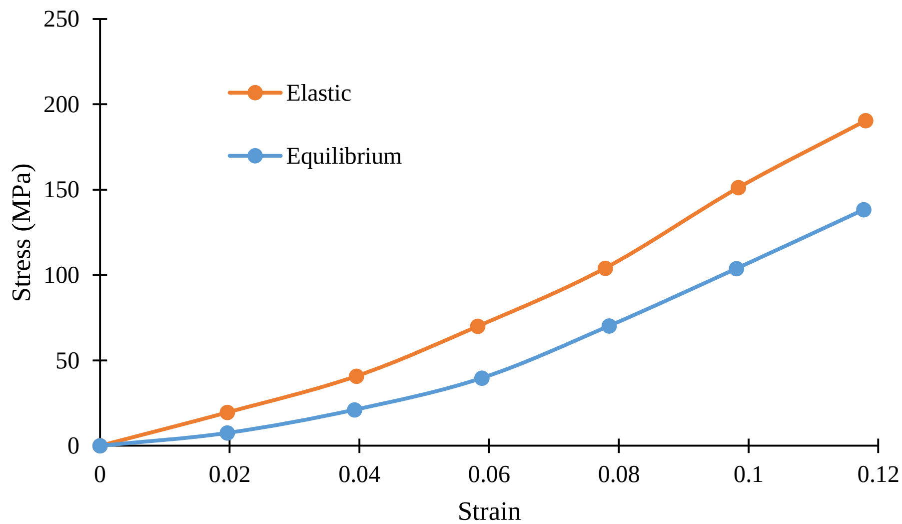

Figure 11: Above is the equilibrium (blue) and elastic (orange) composite curves for the stress strain relationship. This data was collected from the minimum (equilibrium curve) value of the hold and the maximum (elastic) value of the pull for sample five. A larger degree of difference between the elastic and equilibrium curves is evident within the toe region but with less difference as the sample entered into the linear region of the material.

3.3 Normalized values

The normalized curves for the stress strain values for each pull were calculated by subtracting each instantaneous stress value by the initial stress value and each instantaneous strain value by the initial strain value. The curve generated for all six pulls for sample five can be found in Figure 12. The curve demonstrates a linear or logarithmic like behavior across all of the pulls. Also, the rate of change during the interval increases across the six pulls with this value increasing with each subsequent pull. The normalized stress-strain pull curves for the other samples can be found in the appendices.

The normalized curve for the stress-time values for each hold were calculated by dividing each instantaneous stress value by the initial stress value of the hold and subtracting each instantaneous time value by the initial time of the hold. The results for the equilibrium normalized stress-time response for sample five can be found in Figure 13. There is exponential decay with asymptotic behavior found across all six holds. However it appears that this asymptote, or amount of total stress reduction, increases across the six holds. The normalized stress-time hold curves for the other samples can be found in the appendices.

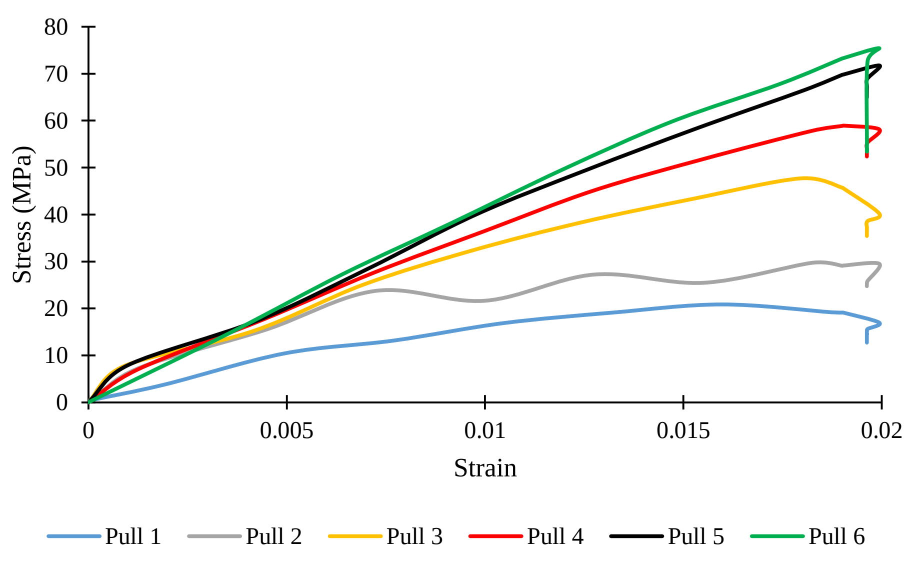

Figure 12: The Figure above depicts the normalized curve of the stress-strain relationship for each pull of sample five. The initial strain and stress were subtracted from the instantaneous values for every point within the interval. The Figure above displays the strain relationship as the rate of change increases between each strain interval. Also, the relationship is clearly not exponential and appears to resemble more of a logarithmic or linear relationship between stress and strain for each pull.

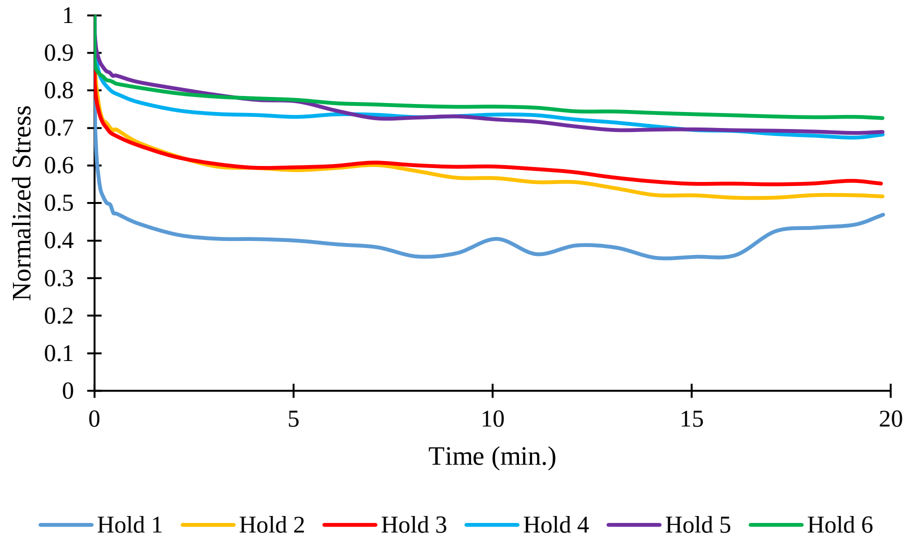

Figure 13: The above Figure displays the normalized values for stress and time within each hold for sample five. The normalized stress was calculated by dividing the instantaneous stress for all points by the initial stress. The time values were normalized by subtracting each instantaneous point by the initial time for each hold. This appears to demonstrate a change in viscoelastic response at different strain points.

3.4 Statistical Testing

For the statistical analysis that was conducted, the normalized data was used for both the stress-strain and stress-time analysis. This data was modeled in a nonlinear format to compare the adjustments that arise from each interval. The values for the coefficients for the pull data can be found in Table 3. These values represent the difference in the rate of change of stress-strain across the six holds and accounts for all seven samples. When subjected to a parameter test to determine significance between the adjustments for each pull, it was found that only four pulls were not different to each other. The adjustments from the mean for Pull 2 and Pull 3 were found to be non-significant with regards to each other (p-value=0.411). The adjustments from the mean for Pull 4 and Pull 5 were also found to be non-significant with regards to each other (p-value=0.110). A Table of the p-values for comparison of each adjustment to each other and the mean can be found in the appendices.

Table 3: The Table below depicts the mean rate of change (B1) for each pull for all of the samples.

	B1 (MPa)
Pull 1	669.74
Pull 2	1328.30
Pull 3	1516.68
Pull 4	2193.88
Pull 5	2444.90
Pull 6	3243.23

In order to statistically model the hold data, a nonlinear framework was used with an exponentially decaying function with asymptotic behavior. Each hold added as an adjustment to the coefficients of the model and the results for each hold can be found in Table 4. The asymptotic value for each hold is indicated by (B1). Using a parameter test for each adjustment, it was found that the asymptote was significantly different across the hold besides for the following: Hold 3 and Hold 6 (p-value=0.06), Hold 4 and Hold 6 (p-value=0.75), and Hold 5 and Hold 6 (p-value=0.71). An adjustment variable was included in the nonlinear model (B2). After using a parameter test to determine whether the adjustments for this value for each of these values that they were significantly different with the exception of: Hold 1 and Hold 2 (p-value=0.08), Hold 3 and Hold 4 (p-value=0.08), Hold 3 and Hold 6 (p-value=0.20), Hold 4 and Hold 5 (p-value=0.55), Hold 4 and Hold 6 (p-value=0.60), Hold 5 and Hold 6 (p-value=0.81). The rate of exponential decay was modeled as the coefficient (B3) for each holding pattern. Using a parameter test to determine significant adjustments in the coefficients between the Holds. There was found to be a significant difference between these adjustments for all the Holds except for the following: Hold 2 and Hold 4 (p-value=0.86), Hold 2 and Hold 5 (p-value=0.25), Hold 3 and Hold 4 (p-value=0.18), Hold 3 and Hold 5 (p-value=0.59), Hold 4 and Hold 5 (p-value=0.43). A Table for all of the p-values and the relationships between the adjustments for each coefficient between each other can be found in the appendices.

Table 4: The Table below depicts the modeled behavior for the stress time relationship for each hold. (B1) the modeled asymptotic value, (B2) the modeled offset value, and (B3) the exponential decay rate for each pull.

	B1 (MPa)	**B2 (MPa)**	**B3**
Hold 1	0.681	0.315	25.7
Hold 2	0.788	0.214	54.6
Hold 3	0.751	0.237	9.35
Hold 4	0.683	0.272	7.71
Hold 5	0.729	0.231	6.02
Hold 6	0.720	0.256	13.2

4 Discussion

The Maxwell model is generally accepted as the method by which stress, strain, and time cane be related to each other in viscoelastic materials. However, one critical assumption of this model is that there are no other dependencies of stress and strain on these innate properties. This assumes superposition of the material, or that the total deformation of a material is the additive effect of a solid and a fluid.

$$\varepsilon_t = \varepsilon_F + \varepsilon_S \tag{4}$$

$$\frac{d\varepsilon}{dt} = \frac{\sigma}{\eta} + \frac{1}{E}\frac{d\sigma}{dt} = 0 \tag{5}$$

This then can be further expounded to a more general model:

$$\sigma = \sigma_o e^{-\frac{E}{\eta}t} \tag{6}$$

However based upon these findings, it is apparent that superposition of a tendon cannot be assumed for viscoelastic testing. This is because the asymptotic values that are present at all of the strain intervals for the holding. All of this values are also non-zero for these intervals as well indicating that that there is another parameter that is needed to fully describe the viscoelasticity of biological materials. This assumption also takes into account that the rate of change with respect to stress-strain are linear and therefore constant. For tendon, it is well known that this is not the case on the intervals that are of interest as rate of change of the stress-strain curve is not linear in the toe region.

The original intent of this work was to investigate a testing method equilibrium tensile testing on a highly collagenous material of rat tail tendon. This was achieved using seven samples with six designated strain points for relaxation of the material. The use of instantaneous testing allowed for the calibration of the strain intervals for the equilibrium testing. This also made possible comparison between the instantaneous response and the equilibrium response of the samples. This form of testing revealed basic viscoelastic tendencies of the material to relax in an exponentially decaying fashion during the holds. Meanwhile, there was an initial stiffening response of the material during the pull portions of the tests. This response was thought to be more of an exponential growth model and may have arisen due to properties of "crimp" as it hardens.

It was found that when the curves were normalized and compared using statistical analysis that the material appeared to show difference across all of the strain points. This may indicate that there is a relationship between the viscoelastic properties and that of the strain for a given sample of tendon. This could primarily be see with the asymptotic values that were generated from the models. Because none of these values were significant and the adjustments were also significant it may be seen that the principle of superposition may not apply to rat tail tendon. However, due to the loading conditions and the lack of a means to hydrate the material during the tensile test, this may be due to an effect of material hardening over time [19]. This may also result may continuously be exacerbated by the stretching of the material and the flow of the fluid within the material across the newly elongated sample.

Stiffening of the material was also observed with the different pulling portions of the equilibrium testing. The stress-strain relationship should be described in more of an exponential gain due to the transition from a lax state to a tensile one. The testing of the tendon samples seemed to indicate more of a linear or even a logarithmic relationship between stress and strain for each pull in the equilibrium test. This behavior could again be due to further stiffening due to drying of the tendon samples during the testing. However, this explanation does not account for the initial pull of each sample displaying such behavior as well. This behavior could also be due to the tendon contracting further during the relaxation stages and a larger load being necessary to elongate the material at first. As the material is elongated, the rate of change of stress with respect to strain reaches a limiting value and proceeds to exhibit more of a linear behavior. The modeling of this type of limit is a factor that could be further investigated in future studies to account for this behavior.

When testing using the equilibrium approach, there appeared to be phasic difference between the purely elastic and the equilibrium curves. There was a clear difference in the elastic and equilibrium curves at the outset of test in the toe region of the stress-strain curve for highly collagenous materials such as tendon. This offset could be due to the elongation of the crimp structures within the microfibrils of the tendon samples. This may be because the equilibrium curve is used because of the energy-storing properties of the material. The intent of the crimp structures is to allow for further laxity of the material. However this is lost in the linear region of the material as the rate of change between stress and strain of the tendon samples. This may be due to the elastic-like properties of tendon within the linear region with the crimp being fully stretched out. With this in mind, the offset between the elastic and equilibrium composite curves is a value for which can further studied at the transition point.

5. Conclusion

Based upon the data from the equilibrium testing method from rat tail tendon, there appears to be a potential strain relationship with regards to the viscoelastic properties of the tendon fascicles. This relationship may rule out the assumption of superposition with regards to modeling viscoelasticity and consideration of a new constant or strain dependent relationship must be undertaken in future studies. This relationship could also be seen in the stiffening response of the tendon samples during the pulling periods of the equilibrium tests. These pulling patterns also exhibited unusually linear or even logarithmic behavior across the samples. The energy storing discrepancies between the elastic and equilibrium methods could be seen primarily through the elongation of the toe region and less so within the linear region. This may indicate a phasic response of tendon with the linear region acting as a proxy for elasticity and the toe region displaying more of the viscoelastic properties of tendon.

As has been stated previously, there are definitely some limitations to the experimental procedure that should be improved in future testing. The primary factor of interest is the problem of hydration of the sample during the equilibrium tests. Due to the prolonged nature of this testing approach, the tendon samples could naturally dry out as a result. They would not completely dry out over the course of the tests due to the behavior of the general stress-time data points in the final stages still exhibiting exponential decay of stress as a function of the material. However, this property would explain the change in the amount of relaxation across the six strain holds.

The type of instrumentation used for the procedure could also produce error in the data as well. The first being noise observed in the load data obtained by the loading cell. Because these samples are very small and experience relatively small loads across the testing paradigm, the use of such a large loading cell could result in a high degree of error. However, this should have been corrected to some extent with the use of time average data during the holds to remove oscillation of the data. The type of load cell could have also resulted in noise in the elongation data as well. This was seen in the non-constant values for the strain points because of machine calibration during the test to achieve an elongation value that was approximate to what was programed.

The dimensional measurements could have been another source of error with respect to cross-sectional area and gauge length. The cross-sectional area was assumed to being approximately a square and width of the sample was assumed to be equal to the thickness of the tendon samples. This may potentially not be entirely true for every tendon fascicle as there may be variation across samples. Also, samples were difficult to properly position for measurement of the width under the micrometer. This may result in a greater error than that of the resolution of the micrometer (0.005 mm). Since this tendon is considered a soft tissue there may have been a risk of shearing that may have occurred during the test. However, this effect may be considered minimal because of the large aspect ratio of the tendon samples. The gauge length of the material could also be another source of error for each specimen because of a balance is needed to keep the tendon samples taut but also not to pre-load them and affecting with the data. This would affect the gauge length as this value was recorded within the load cell.

Because the samples were relatively small in comparison to the fixture that was used, additional noise could have been incorporated into the results. On top of the large discrepancy in size, the tendon samples were also wet and were not easy to grip for tensile testing. Because of these factors, many of the tendon samples failed because of slippage during testing. This was due to the adhesive not properly curing in time to provide a solid surface for which the tendon fascicles could be secured. Because of this occurrence in preliminary testing, the strain rate had to be reduced to the value used of 2.5 mm/sec. This change in strain rate from higher rates may draw into question whether the instantaneous samples were instantaneous. However this rate is fast enough to properly represent the data in an elastic manner for both the pulls and the instantaneous tests [20, 21].

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APPENDICES

Appendix A: Global Stress-Strain Curves

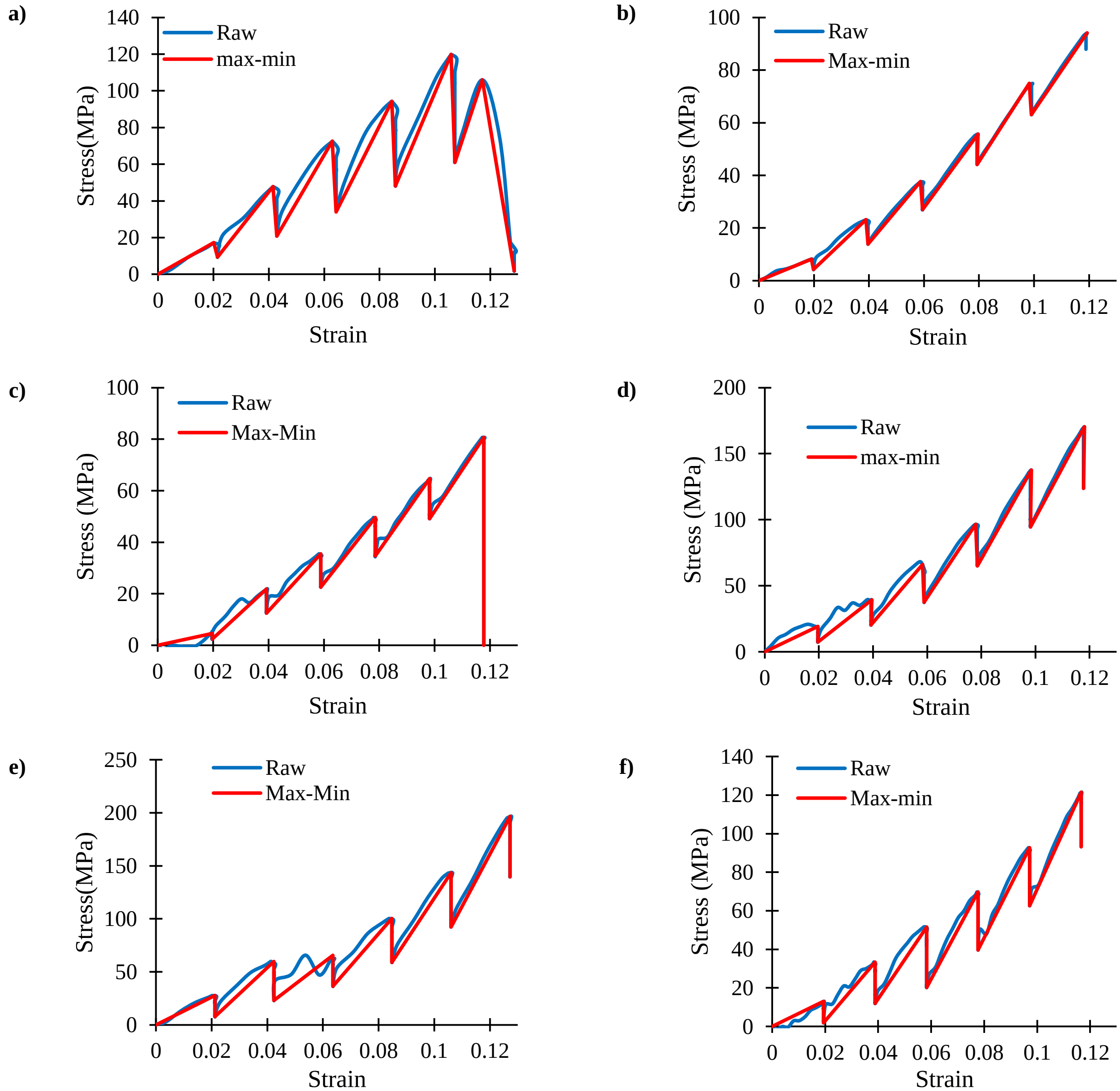

Figure A.1: The equilibrium stress-strain curve with six strain points at intervals of 2% strain. The test was conducted in tension at 2.5mm/sec uniaxial in a loading cell. The above Figure is of a) Sample one, b) Sample two, c) Sample four, d) Sample five, e) Sample six, f) Sample seven. The depicted raw data (blue) and composite curve of the minimum values of the hold and maximum values of the pull (red). These curves demonstrate the initial stiff response of each pull and the limit of the modulus between each pull as the strain increases.

Appendix B: Global Stress-Time Curves

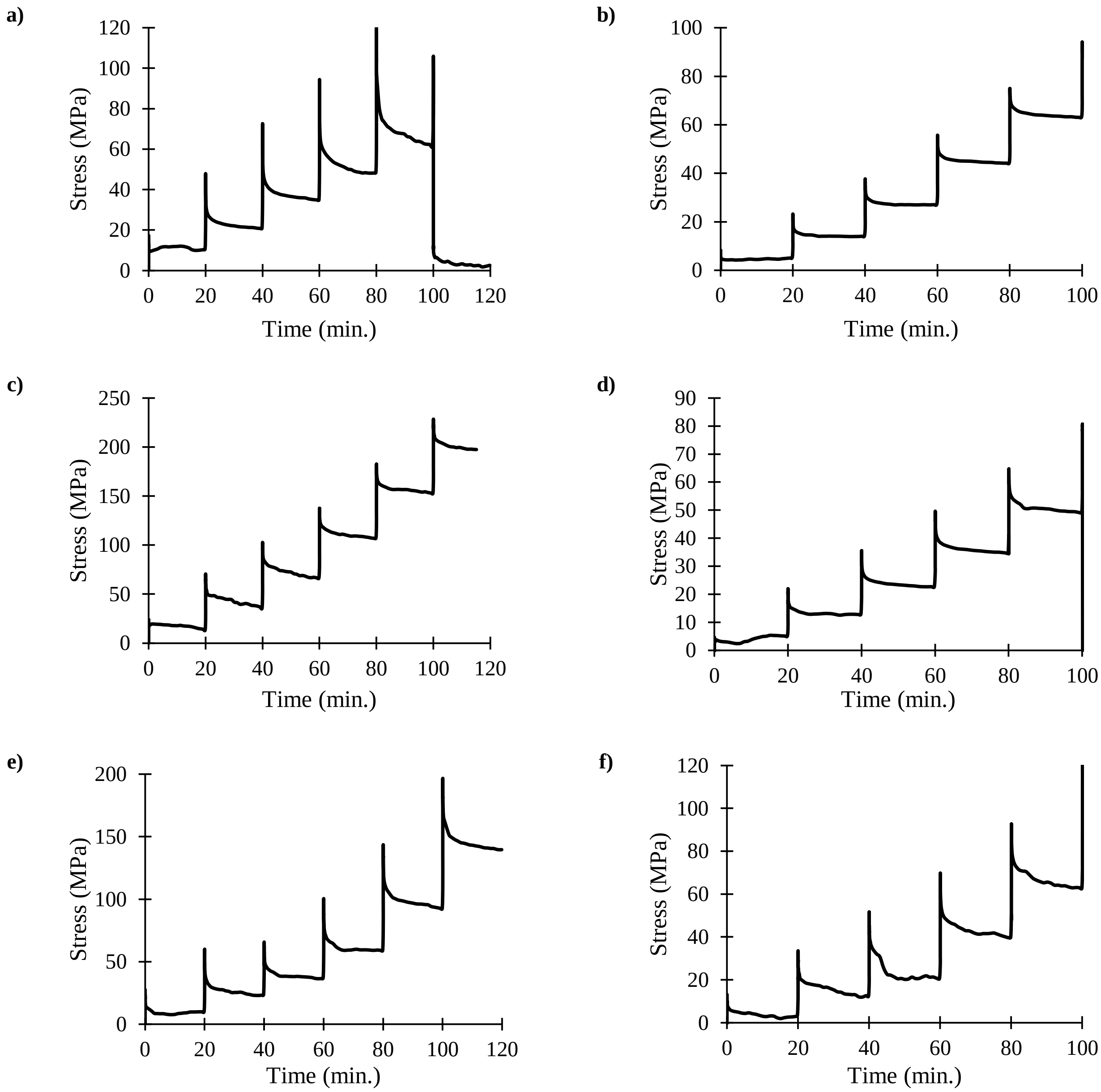

Figure B.1: The equilibrium time-stress curve with six strain points at intervals of 2% strain. The test was conducted in tension at 2.5 mm/sec uniaxial in a loading cell for pulls and the holds occurring at each strain point for 20 minutes. The Figure above depicts the curve for a) Sample one, b) Sample two, c) Sample three, d) Sample four, e) Sample six, f) Sample seven. This Figure displays the viscoelastic response of rat tail tendon across six different strain points.

Appendix C: Normalized Stress-Strain Curves for Pull Data

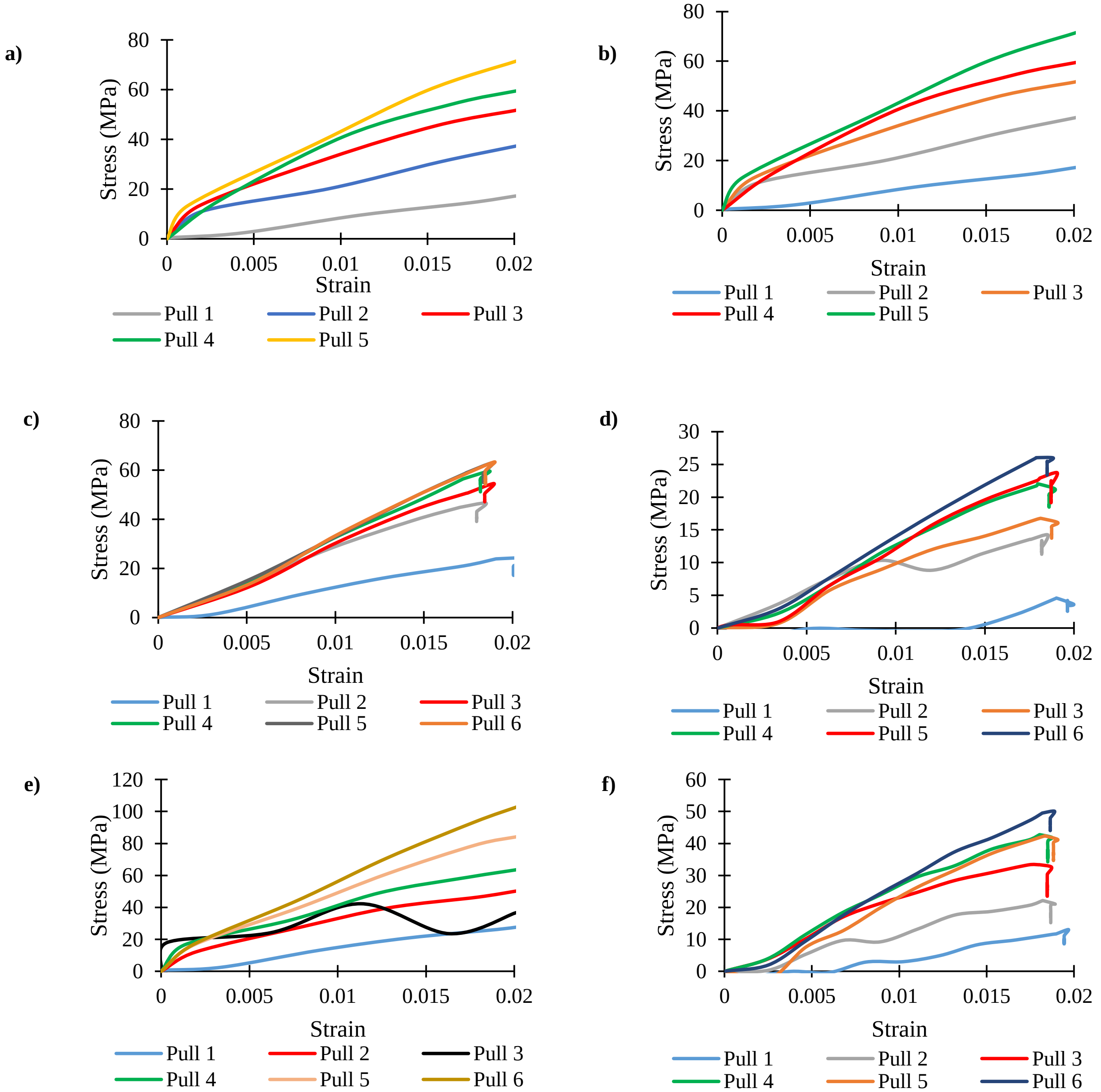

Figure C.1: The Figure above depicts the normalized curve of the stress strain relationship for each pull for a) Sample one, b) Sample two, c) Sample three, d) Sample four, e) Sample six, f) Sample seven. The initial strain and stress were subtracted from the instantaneous values for every point within the interval. The Figure above displays the strain relationship as the rate of change increases between each strain interval. Also, the relationship is clearly not exponential and appears to resemble more of a logarithmic or linear relationship between stress and strain for each pull.

Appendix D: Normalized Stress-Time for Hold Data

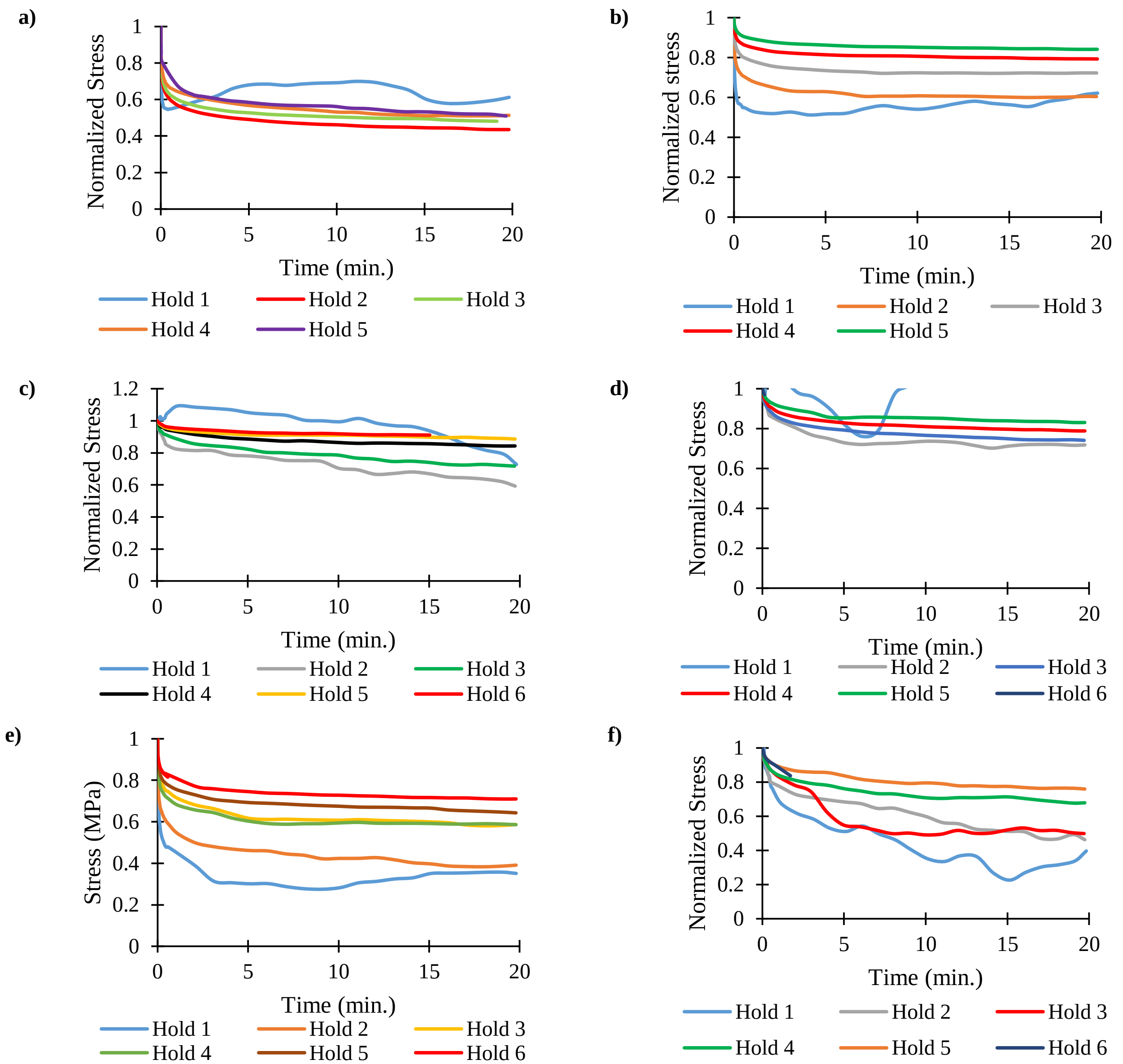

Figure D.1: The above Figure displays the normalized values for stress and time within each hold for a) Sample one, b) Sample two, c) Sample three, d) Sample four, e) Sample six, f) Sample seven. The normalized stress was calculated by dividing the instantaneous stress for all points by the initial stress. The time values were normalized by subtracting each instantaneous point by the initial time for each hold. The above Figure appears to demonstrate a viscoelastic properties with the presence of an asymptote for every strain value and this value increasing for each strain interval.

Appendix E: Comparison of Equilibrium and Elastic Curves

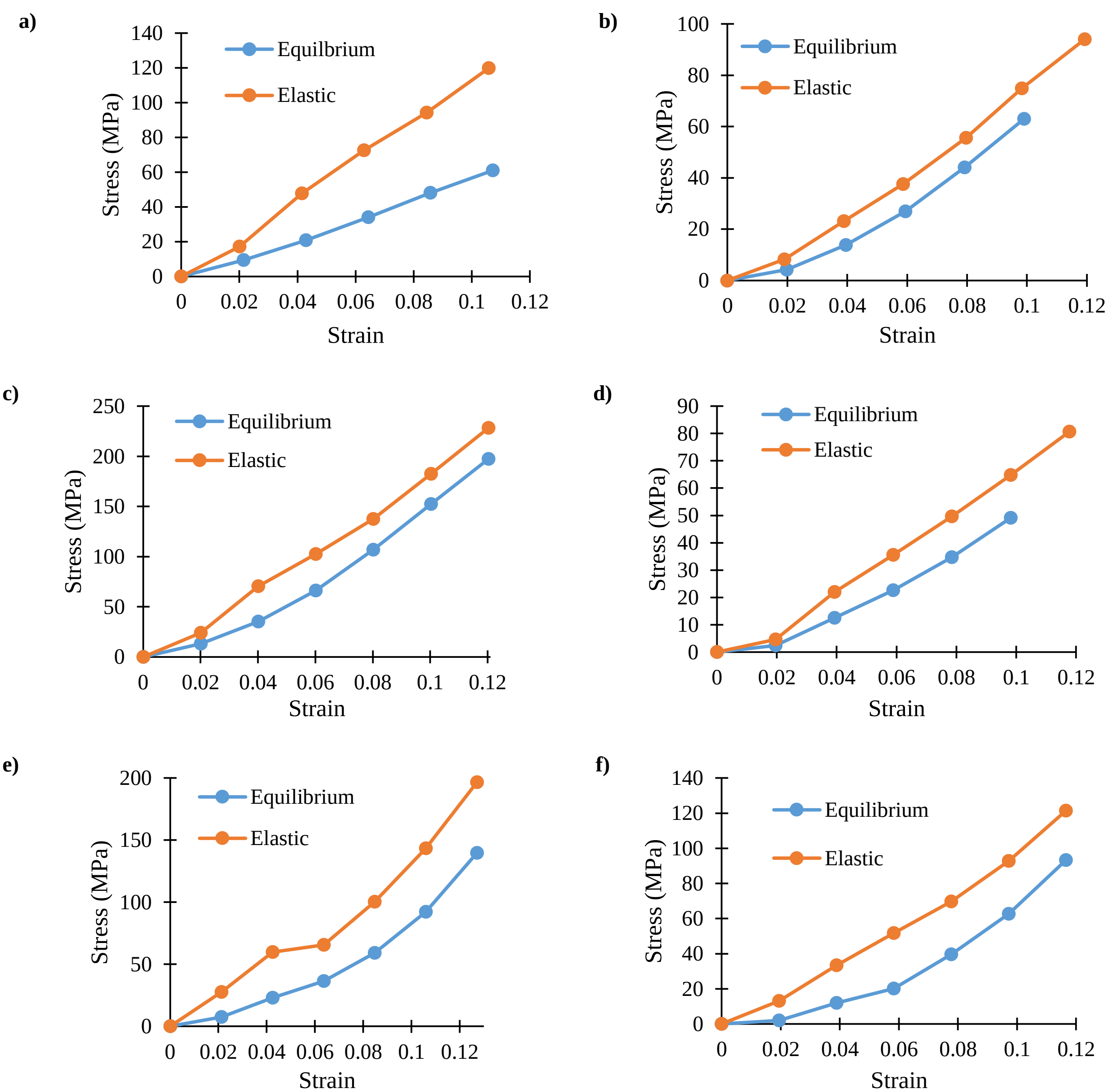

Figure E.1: Above is the equilibrium (blue) and elastic (orange) composite curves for the stress strain relationship. This data is collected from the minimum (equilibrium curve) value of the hold and the maximum (elastic) value of the pull. The above Figure is of a) Sample one, b) Sample two, c) Sample three, d) Sample four, e) Sample six, f) Sample seven. The Figure above demonstrates high degree of difference between the elastic and equilibrium curves within the toe region but similar rates of change as the materials enter into the linear region of the material.

Appendix F: p-values for Statistical Model of Pulls

Table F.1: Table of the p-values for the adjustment of the slope parameter between pulls for all subjects.

	Pull 1	Pull 2	Pull 3	Pull 4	Pull 5	Pull 6
0	1.15e-04	.0116	6.60e-04	3.40e-09	3.37e-12	5.50e-21
Pull 3	-	.475	-	-	-	-
Pull 4	-	0.0015	0.00942	-	-	-
Pull 5	-	3.52e-05	3.21e-04	0.34684	-	-
Pull 6	-	2.83e-11	4.31e-10	0.00023	0.00468	-

Appendix G: p-values for Statistical Model of Holds

Table G.1: The p-values for the adjustment variables generated for each hold for B1. This value represents the asymptotic tendency of the model.

	Hold 1	Hold 2	Hold 3	Hold 4	Hold 5	Hold 6
0	0	3.94e-56	2.55e-18	.843	6.23e-07	8.71e-07
Hold 3	-	1.10e-07	-	-	-	-
Hold 4	-	1.00e-42	3.28e-15	-	-	-
Hold 5	-	7.99e-13	0.0164	2.78e-06	-	-
Hold 6	-	5.08e-24	7.26e-05	7.93e-06	0.337	-

Table G.2: The p-values for the adjustment variables generated for each hold for B2. This value represents an exponential adjustment variable for the model.

	Hold 1	Hold 2	Hold 3	Hold 4	Hold 5	Hold 6
0	1.27e-41	0.00306	0.00954	1.42	0.00372	0.0575
Hold 3	-	0.4634	-	-	-	-
Hold 4	-	0.0621	0.190	-	-	-
Hold 5	-	0.5667	0.824	0.106	-	-
Hold 6	-	0.2018	0.513	0.563	0.369	-

Table G.3: The p-values for the adjustment variables generated for each hold for B3. This value represents the rate of exponential decay for the model.

	Hold 1	Hold 2	Hold 3	Hold 4	Hold 5	Hold 6
0	4.93e-09	0.0974	4.56e-04	7.08e-05	1.25e-05	0.022
Hold 3	-	0.00757	-	-	-	-
Hold 4	-	0.00554	0.3880	-	-	-
Hold 5	-	0.00404	0.0711	0.250	-	-
Hold 6	-	0.01773	0.0964	0.0157	0.00199	-

Appendix H: p-value Comparison of Elastic and Equilibrium Curves

Table H.1: p-values comparing each period between the equilibrium and elastic curves across samples.

Period	p-value
1	0.0584
2	0.0124
3	0.3324
4	0.4837
5	0.4424
6	0.7030

Table H.2: p-values comparing periods to each other within the elastic curve.

	1	**2**	**3**	**4**	**5**	**6**
1	-	0.0536	0.433	0.04858	0.00139	3.02e-06
2	0.0536	-	0.252	0.96661	0.20547	0.0025
3	0.433	0.252	-	0.23505	0.01588	6.32e-05
4	0.04858	0.96661	0.23505	-	0.22085	0.00281
5	0.00139	0.20547	0.01588	0.22085	-	0.05193
6	3.02e-06	0.0025	6.32e-05	0.00281	0.05193	-

Table H.3: p-values comparing periods to each other within equilibrium curve.

	1	**2**	**3**	**4**	**5**	**6**
1	-	0.186	0.0875	0.00155	1.55e-05	4.80e-09
2	0.186	-	0.699	0.06535	0.00271	2.28e-06
3	0.0875	0.699	-	0.14531	0.00898	1.10e-05
4	0.00155	0.06535	0.14531	-	0.247	0.00161
5	1.55e-05	0.00271	0.00898	0.247	-	0.0301
6	4.80e-09	2.28e-06	1.10e-05	0.00161	0.0301	-